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Protective Effect of γ-Glutamylcysteinylethyl Ester on Dysfunction of the Selenium-Deficient Rat Heart

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ABSTRACT. We investigated the protective effect of intracellular GSH against cardiac dysfunction in selenium (Se)-deficient neonatal rats and cultured fetal rat myocytes. A Se-deficient diet with or without daily subcutaneous injections of γ-glutamylcysteinylethyl ester (γ-GCE) (a membrane-permeating GSH precursor) was given to rats from gestation day 4 via the dam to postnatal day 14. Se deficiency induced a 62% incidence of electrocardiographic abnormalities such as sinus arrhythmias or extrasystole, a 63% reduction in dP/dt in the left ventricle, and an increase in thiobarbituric acid reacting substances (TBARS), but no ultrastructural cardiac lesions were observed. Administration of γ-GCE increased the intracellular GSH concentration ($[GSH]_i$) of both neonatal rat hearts and cultured fetal rat cardiac myocytes. γ-GCE-like sodium selenite prevented the cardiac dysfunction and the TBARS increment. γ-GCE also prevented H_2O_2 toxicity in the cultured myocytes. The V_{max}, but not the K_m, for GSH of Se-dependent GSH peroxidase (Se-Gpx) activity in Se-deficient rat heart homogenates was one-third that of normal rat heart homogenates. Although γ-GCE did not affect the Se-Gpx V_{max} and K_m for GSH, it did induce a substantial and significant increase in $[GSH]_i$, which was postulated to increase the velocity of H_2O_2 decomposition by Se-Gpx activity 1.6-fold. These data suggest that the increase in $[GSH]_i$ may have played a role in preventing the TBARS increase and cardiac dysfunction in Se-deficient rats. BIOCHEM PHARMACOL **57**;8:955–963, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. selenium; glutathione; heart; cultured cardiac myocytes; Se-dependent glutathione peroxidase; thiobarbituric acid reacting substances

Oxygen radicals and oxidants are involved in several kinds of cardiac injury, including ischemia–reperfusion injury [1, 2]. The cardiotoxic effects of oxygen radicals and oxidants, however, can be counteracted by the cellular antioxidant defense system. Se-Gpx¶ (EC 1.11.1.9), superoxide dismutase (EC 1.15.1.1), and catalase (EC 1.11.1.6) are the primary antioxidant enzymes involved in the direct elimination of active oxygen radicals and oxidants. The eliminating activity of Se-Gpx in the human myocardium is 10 to 15 times higher than that of superoxide dismutase and catalase [3]. Se-Gpx is a key enzyme in the reduction of several organic hydroperoxides and hydrogen peroxide, the latter being the major product of the dismutation of superoxide anion by superoxide dismutase [4, 5]. Se-Gpx has a metal cofactor, Se. This essential trace element is incorporated in many eukaryotic and prokaryotic selenoproteins such as selenocysteine and is a prerequisite for the expression of these proteins [6, 7]. Se is a structural component of the active site of Se-Gpx [8], and Se deficiency is associated with a decrease in Se-Gpx activity [9, 10] and increased lipid peroxidation [11]. Keshan disease, which causes juvenile cardiomyopathy and is endemic in China, is associated with several interacting factors, including nutritional deficiencies (primarily Se, but also vit. E and polyunsaturated fatty acids) and an infectious agent (coxsackievirus B) [12, 13]. In 1979, Chinese scientists carried out an extensive Na_2SeO_3 (sodium selenite) supplementation trial, which provided convincing evidence that Se could prevent the disease [14]. Indeed, Se is an essential nutrient for humans and experimental animals, but Se and Na_2SeO_3 also possess toxic attributes, which are evident from their carcinostatic activity [15, 16].

Recently, it was demonstrated that the membrane-permeating GSH precursor, γ-GCE, is transported into isolated GSH-depleted rat hepatocytes more readily than GSH itself and that the SH-containing ester, after being

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¶*Abbreviations:* ECG, electrocardiography; γ-GCE, γ-glutamylcysteinylethyl ester; $[GSH]_i$, intracellular glutathione concentration; LV, left ventricle; Se(−) diet, selenium-deficient diet; Se(−) diet + γ-GCE, γ-GCE-injected selenium-deficient diet; Se(−) diet + GSH, GSH-injected selenium-deficient deit; Se(−) diet + Na_2SeO_3, Na_2SeO_3-supplemented selenium-deficient diet; Se-Gpx, selenium-dependent glutathione peroxidase; TBARS, thiobarbituric acid reacting substances; and vit., vitamin.

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hydrolyzed by esterase, is converted to GSH by glutathione synthetase [17]. γ-GCE can also protect against ischemia–reperfusion cell injury, and the protective effect is much greater for γ-GCE than for GSH [18–20]. We reported previously that myocytes from fetuses of pregnant mice fed a Se(−) diet, relative to those from pregnant mice fed a normal diet, were more susceptible to H_2O_2 toxicity, and their Se-Gpx activity was about 45% less [21]. Furthermore, preloading cultured mouse cardiac myocytes with γ-GCE increases both $[GSH]_i$ and H_2O_2 resistance [21]. From those observations, we hypothesized that the heart dysfunction induced by Se depletion may be attenuated by increasing the $[GSH]_i$, the H-donating substrate for Se-Gpx.

In the present study, we investigated whether rats fed a Se(−) diet show cardiac dysfunction, and if so, whether and how this dysfunction is attenuated by increasing $[GSH]_i$. We used fetal and neonatal rats to test the hypothesis because they show relatively low esterase activity [17], meaning that γ-GCE administered *in situ* would not be de-esterified before reacting with heart tissues.

MATERIALS AND METHODS

Agents

H_2O_2, Na_2SeO_3, 5,5′-dithiobis(2-nitrobenzoic acid), and fibronectin from bovine plasma were purchased from the Wako Chemical Co. Trypsin (1:250) was obtained from Difco Labs. Trypsin inhibitor (Type 1-S) and GSH (reduced form) were obtained from the Sigma Chemical Co. β-NADPH and GSH reductase (from yeast) were purchased from the Oriental Yeast Co. All reagents were of analytical grade. γ-GCE was provided by the Teijin Institute for Biomedical Research, Tokyo, Japan.

Animals and Their Treatment

Specific-pathogen-free rats pregnant for 3 days (Wistar strain, 146–178 g) were purchased from the SLC Co. and housed individually in plastic cages with hardwood bedding (SLC autoclaved Softchip). To prevent contamination with trace amounts of Se, the water they were fed *ad lib.* was distilled (WQ500, Millipore Corp.). Animals were kept in a temperature-controled environment with an automatic 12-hr light/dark cycle (8:00 a.m. to 8:00 p.m.). All treatment was in accordance with a protocol approved by the Animal Care Committee of Kobe Gakuin University.

The rats were given a standard laboratory diet (Labo MR Stock, SLC Co.) for one day and then divided into five groups of four to thirteen: 1st, standard diet; 2nd, Se(−) diet; 3rd, Se(−)diet+Na_2SeO_3; 4th, Se(−) diet + GSH; and 5th, Se(−) diet + γ-GCE. All groups had approximately the same mean body weight. The groups were fed either a standard diet or a torula yeast-based semisynthetic Se(−) diet (CLEA Japan, Inc.), torula yeast 40%, granule sugar 46.5%, purified lard 5%, DL-methionine 0.5%, purified vit. mix. 1%, purified mineral mix. 7%) containing either 0.5 ppm (Se(+) diet) or <0.002 ppm (Se(−) diet). The purified vit. and mineral mixtures of the Se(−) diet were as follows: vit. mixture (IU or mg/100 g of Se(−) diet), vit. A 1200 IU; vit. D_3 2400 IU; vit. E 10; vit. K_3 0.3; vit B_1 1.5; vit. B_2 1.56; vit. B_6 1.02; biotin 0.01; calcium DL-pantothenate 4; ρ-aminobenzoic acid 10.15; nicotinic acid 10.15; inositol 15; folic acid 0.2; choline chloride 300; vit. B_{12} 0.005; corn starch 628.22; mineral mixture (mg/100 g of Se(−) diet), $CaCO_3$ 1355.4; KH_2PO_4 1730; $CaHPO_4 \cdot 2H_2O$ 1500; $MgSO_4 \cdot 7H_2O$ 800; NaCl 600; $FeC_6H_5O_7 \cdot 5H_2O$ 190; $5ZnO \cdot 2CO_2 \cdot 4H_2O$ 6; $CuSO_4 \cdot 5H_2O$ 1.26; $CoCl_2 \cdot 6H_2O$ 0.4; $Ca(IO_3)_2$ 1.54; $MnSO_4 \cdot 4H_2O$ 15.4; corn starch 800.

The Se(−) diet + GSH and Se(−) diet + γ-GCE groups were fed the Se(−) diet and injected subcutaneously with GSH or γ-GCE (0.5 mL of isotonic saline containing 350 μmol GCE or 350 μmol γ-GCE per g body weight), respectively, twice a day (8:30 a.m. and 7:30 p.m.). The Se(−) diet + Na_2SeO_3 group was given the Se(−) diet supplemented with Na_2SeO_3 (1 μg/g Se(−) diet) and was injected subcutaneously with 0.5 mL saline twice a day as described above. The rats born of mothers that had been treated by γ-GCE injection were similarly injected from day 3 after birth to day 14.

Hemodynamic Recordings

SURGICAL PREPARATION. Rats were anesthetized with intraperitoneal injections of sodium pentobarbital (0.06 mg/g of body weight), intubated, and ventilated immediately with room air (0.02 mL/g of body weight) at a rate of 54 strokes/min to maintain arterial blood gases and pH within the normal range. The dP/dt was obtained by canulating directly into the LV with a heparin-filled syringe (0.3 mm inner diameter) connected to an operational amplifier (National Semiconductor LM 324), and readings were recorded on a thermal recorder (Fukuda Denshi Co.).

RECORDINGS OF ECG. The recordings of ECG of standard lead II on 14-day-old rats was recorded on a thermal recorder (Fukuda Denshi Co.).

Measurement of Se Concentration in Heart Tissue

Hearts isolated from neonatal rats were homogenized in 20 vol. of ice-cold saline for 3 min with a polytron homogenizer (PT 10/35, Kinematica Co.). The homogenates were centrifuged at 1000*g* for 5 min and the Se concentration of the supernatant determined by a fluorometric method [22].

Measurement of TBARS

TBARS levels in isolated neonatal rat heart were determined according to a previous report [23]. Hearts weighing 100 to 200 mg were homogenized in 3 mL 1.15% (w/v) KCl for 3 min with a polytron homogenizer. The homogenate (0.6 mL) was mixed with 0.2 mL 8.1% (w/v) SDS, 1.5 mL 20% (w/v) acetate buffer (pH 3.5), and 1.5 mL 0.8% (w/v)

2-thiobarbituric acid and adjusted to 4 mL with distilled water. The mixture was boiled at 95° for 1 hr and quickly cooled in an ice-cold bath. Then, 0.5 mL distilled water was added and the red products of TBARS were extracted by mechanical reciprocal shaking at 80 rpm for 10 min with 2.5 mL of a mixture of *n*-butanol and pyridine (15:1, v/v). The mixture was centrifuged at 1500*g* for 10 min, and the absorbance of the supernatant was measured at 532 nm. The standard used was 1,1,3,3-tetraethoxypropane. The protein content of the homogenates was determined as described previously [24].

Preparation and Culture of Cardiac Myocytes from Rat Fetuses

Heart ventricles were removed from 14-day-old fetuses taken from dams given the Se(−) diet, Se(−) diet + Na_2SeO_3, Se(−) diet + GSH, Se(−) diet + γ-GCE, or standard diet. The tissues were dissociated into single cells by trypsinization [21]. The reaction was stopped by addition of trypsin inhibitor (at a 2 × molar concentration for trypsin), and the cells were plated in plastic Petri dishes (Falcon 3001, Japan Becton Dickinson) precoated with 20 mg/mL fibronectin and cultured in serum-, cysteine-, and methionine-free Eagle's minimum essential medium to avoid any effects of Se and GSH. The dishes were subjected immediately to gyratory shaking for 30 to 60 sec to obtain a large cell sheet in the center of the dish. A 1- to 2-mm-diameter sheet containing at least 10^4 cells was produced after 2 days.

Observation of Beating Cultured Cardiac Myocytes

The beating of cultured cardiac myocytes was observed with an inverted phase-contrast microscope (Olympus IMT-2 or Nikon Diaphot), while the cells were maintained at 37° and pH 7.3 in Eagle's minimum essential medium under a flow of 5% CO_2 in air.

Electrical Field Stimulation

After the spontaneous beating of cultured cardiac myocytes was stopped by H_2O_2 treatment, the cells were stimulated electrically. Two platinum 9 × 1.5 × 0.2-mm electrodes were immersed in bathing medium 10-mm apart, and the cells were stimulated at 1 Hz with an isolator-equipped electronic stimulator (Nihon Kohden SEN-3301 and SS-201J). The duration and amplitude of the applied rectangular pulses were 50 msec and 100 V, respectively.

Measurement of Se-Gpx Activity and $[GSH]_i$

Se-Gpx activity. Neonatal rat hearts were washed out with an ample volume of saline before sampling. The tissue (100–200 mg) was homogenized in 100 vol. of 10 mM sodium phosphate buffer (pH 7.0). The homogenates were centrifuged at 2000*g* for 15 min. The supernatants were re-centrifuged at 105,000*g* for 1 hr, and their supernatants were stored at −80° until assayed.

The Se-Gpx activity was determined using 1.5 mM H_2O_2 as substrate [25]. The reaction mixture was prepared fresh daily and consisted of 10 mM sodium phosphate buffer (pH 7.0), 0.2 mM NaN_3, 200 mM GSH (reduced form), 1 unit/mL GSH reductase, 0.2 mM NADPH, and 100 mL of sample in a total volume of 1.0 mL. The overall reaction was started by adding 1.5 mM H_2O_2. Samples were allowed to incubate for 2 min at 25° before the addition of H_2O_2, which started the reaction. Absorbance at 340 nm was recorded for 3 min, and the extinction coefficient of 6.22 $mM^{-1} \times cm^{-1}$ was used to express the activity as mmol NADPH oxidized/min/mg protein over the linear range.

$[GSH]_i$. $[GSH]_i$ in the heart tissue homogenates was determined by the enzymatic recycling method with GSH reductase and 5,5′-dithiobis(2-nitrobenzoic acid) [26]. γ-GCE had no significant effect on the determination of $[GSH]_i$ [17]. Heart tissues were homogenized in 10 vol. of ice-cold 2.5% (w/v) sulfosalicylic acid and centrifuged at 10,000*g* for 3 min at 4°, and the supernatant was used for the determination of $[GSH]_i$ [21]. In the case of cultured cardiac myocytes, the cells (1×10^7) were removed from the dishes with a rubber policeman (Cell Scraper 11 mm × 220 mm, Iwaki Glass Co.), homogenized, and collected in test tubes; $[GSH]_i$ was then measured by the same method that was used for the homogenates [21].

Statistical Analysis

Results were analyzed by computerized statistical packages (Super-ANOVA, Statview). Statistical significance of differences among means was analyzed using one-way analysis of variance (ANOVA) and the multiple range test. A probability of <0.01 was taken as the level of significance.

RESULTS

Maternal and Neonatal Health in the Se(−) Rats

Compared with the standard diet, the Se(−) diet had no statistically significant effect on the duration of pregnancy or litter size, nor did it impair the body weight gain of pregnant or neonatal rats (Table 1). Each group consumed an equivalent amount of food and water. Several reports have demonstrated that severe Se deficiency is characterized by cardiomyopathy and moderate deficiency by less severe myodegenerative signs such as muscular weakness [27], while rats fed diets from the Keshan area develop liver necrosis [28]. In the present study, however, rats fed the Se(−) diet from fetal day 4 to postnatal day 14 were normal in appearance, behavior, and body weight, although the Se content in heart (Table 1) and whole body tissue was one-third that of rats fed the standard diet. Neither light microscopy nor electron microscopy revealed any severe injury in heart tissue or in cardiac myocytes, suggesting that Se depletion alone does not induce severe systemic injury

TABLE 1. Effect in rats of Se(−) diet on pregnancy, neonates, and Se concentration in neonatal heart tissue

Diet	Body weight (g) of maternal rats		Duration of pregnancy (day)	Rats/Litter	Body weight (g) of neonatal rats		Se content in heart on day 14 after birth (ppm)
	Day 4 of pregnancy	Day 18 of pregnancy			Day 3	Day 14	
Standard	170 ± 7	230 ± 14	20 ± 1	9 ± 2	7 ± 1	21 ± 2	0.09
Se(−)	166 ± 9	237 ± 11	20 ± 2	9 ± 1	7 ± 1	21 ± 3	0.03
Se(−) + Na_2SeO_3*	161 ± 11	239 ± 13	20 ± 1	10 ± 3	7 ± 1	20 ± 3	0.08
Se(−) + γ-GCE injection†	168 ± 7	241 ± 10	20 ± 1	11 ± 2	7 ± 1	22 ± 2	0.02

Results are expressed as means ± SD of four to thirteen experiments, except for Se contents of hearts, which are expressed as mean values derived from a single experiment performed in triplicate (variation <5%).

*Na_2SeO_3 supplementation (1 μg/g of Se(−) diet).

†Subcutaneous injection of γ-GCE (350 μmol/g body weight) twice a day.

such as Keshan disease. Therefore, at least for the experimental parameters assessed, neither maternal nor neonatal health appeared to be compromised by the Se(−) diet. Moreover, no toxic effects of Na_2SeO_3 or γ-GCE were observed during the experimental period. Administration of γ-GCE did not affect the Se content in hearts of rats fed Se(−) (Table 1) or standard diets (0.08 ppm).

Heart Dysfunction Induced by Se Depletion

We first studied whether Se depletion impaired neonatal heart function. Neonatal rats fed the Se(−) diet showed a high incidence of ECG abnormalities such as sinus arrhythmia and extrasystole (Table 2 and Fig. 1). Of the thirteen 14-day-old rats that had been fed the Se(−) diet, five (38%) had sinus arrhythmia and three (23%) extrasystole. Figure 1 showed typical ECG abnormalities of the neonatal rats fed the Se(−) diet. Such ECG changes continued for more than 10 min. In the standard diet and Se(−) diet + γ-GCE groups, in contrast, almost no ECG abnormalities were observed.

The mean neonatal LV dP/dt values (mmHg/sec, means ± SD) were 1042 ± 115 for the standard diet group and 1117 ± 75 for the Se(−) diet + γ-GCE group (Table 2), with no statistically significant difference between them. On the other hand, the LV dP/dt value was significantly lower in the Se(−) diet group than in the standard diet group, suggesting that Se depletion alone caused cardiac dysfunction, and that γ-GCE played an active protective role against it. In addition, no significant lesions were observed by ultrastructural or light (hematoxylin and eosin stain) microscopy in sections prepared from the left ventricle of rats showing ECG abnormalities.

TABLE 2. Electrocardiographic abnormality incidence and LV dP/dt values in neonatal rats from dams fed Se(−) diet

Diet	Incidence of electrocardiographic abnormalities (%)	LV dP/dt (mmHg/sec)
Standard	0/13 (0%)	1042 ± 115
Se(−)	8/13 (62%)	381 ± 79‡
Se(−) + Na_2SeO_3*	0/5 (0%)	N.D.
Se(−) + γ-GCE injection†	1/13 (8%)	1117 ± 75

Results are expressed as means ± SD of four experiments. N.D., not determined. The LV dP/dt differed among the three groups (one-way ANOVA, $P < 0.005$).

*Na_2SeO_3 supplementation (1 μg/g of Se(−) diet).

†Subcutaneous injection of γ-GCE (350 μmol/g body weight) twice a day.

‡Significantly different from standard diet (LSD post-ANOVA test, $P < 0.001$).

Effects of γ-GCE on $[GSH]_i$ and Se-Gpx Activity in Neonatal Rat Heart Homogenates

We next measured $[GSH]_i$ and Se-Gpx activity in heart homogenates prepared from 14-day-old rats that had been

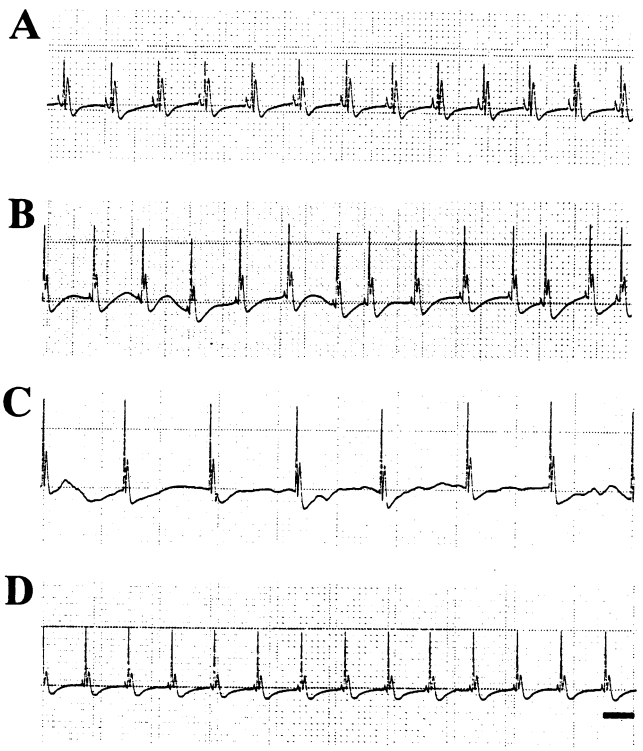

FIG. 1. Tracings showing ECG abnormalities induced by the Se(−) diet. (A) A tracing from a rat fed the standard diet; heart rate was 312 beats/min. (B) and (C) Tracings from rats fed the Se(−) diet; characteristic ECG abnormalities show sinus arrhythmia (B) and extrasystole (C). The decrease in heart rate was transient. (D) A tracing from a rat given the Se(−) diet + γ-GCE; ECG was normal. Time calibration represents 0.125 sec.

TABLE 3. Effects of γ-GCE on $[GSH]_i$ and Se-Gpx kinetics in heart tissue from 14-day-old rats

Diet	$[GSH]_i$ (nmol/mg protein)	Se-Gpx kinetics	
		V_{max} (unit/mg protein/min)	K_m for GSH (mM)
Standard	22.8 ± 3.4	21 ± 0.4	11.8 ± 0.4
Se(−)	20.5 ± 2.9	7 ± 0.3‡	11.1 ± 0.3
Se(−) + γ-GCE injection*	28.4 ± 2.5†	7 ± 0.2‡	11.1 ± 0.4

$[GSH]_i$ indicates the intracellular concentration of total (oxidized and reduced) glutathione.

ANOVA: $P < 0.005$ standard diet vs Se(−) diet + γ-GCE in $[GSH]_i$ and $P < 0.0001$ standard diet vs Se(−) diet or Se(−) diet + γ-GCE in V_{max} of Se-Gpx activity. Results are expressed as means ± SD of four experiments.

*Subcutaneous injection with γ-GCE (350 μmol/g of body weight of a rat) twice a day.

†$P < 0.05$ by LSD.

‡$P < 0.0001$ by LSD.

given the standard diet, Se(−) diet or Se(−) diet + γ-GCE. The concentration of total glutathione (oxidized form [GSSG] + reduced form [GSH]) in rats given the Se(−) diet + γ-GCE increased significantly about 1.3 times that of rats fed the standard diet (Table 3); however, there were no significant differences between the standard and Se(−) diet groups. About 85% of total glutathione was in reduced form in the homogenates from the standard diet, Se(−) diet, and Se(−) diet + γ-GCE groups. This indicated that the administered γ-GCE entered the cells and was converted to GSH; it also suggested that the high GSH levels may have contributed to the γ-GCE-induced cardioprotective effect.

Next, we carried out a kinetic analysis of Se-Gpx to obtain the apparent K_m and V_{max} for GSH. A Lineweaver–Burk plot indicated that the K_m values (11.1 ~ 11.8 mM) for GSH were similar for the standard diet, Se(−) diet, and Se(−) diet + γ-GCE groups (Table 3 and Fig. 2). On the other hand, the V_{max} of the enzyme from the Se(−) diet and Se(−) diet + γ-GCE groups was about one-third that of the enzyme from the standard diet group.

These observations suggested that γ-GCE administered to rats fed the Se(−) diet did not affect the V_{max} and K_m for Se-Gpx oxidation of GSH, and that γ-GCE may exert a cardioprotective effect by increasing the concentration of the oxidizable substrate ($[GSH]_i$) for Se-Gpx.

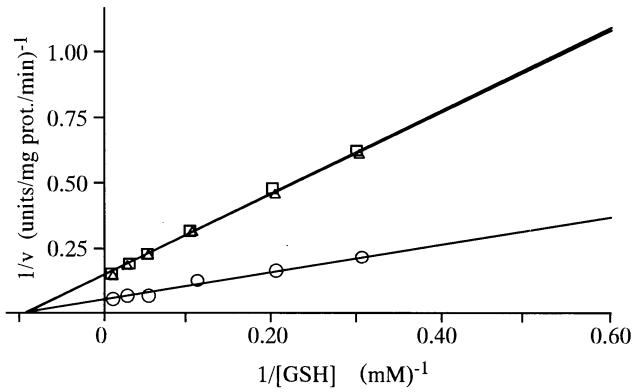

FIG. 2. Lineweaver-Burk plots of Se-Gpx activity. Se-Gpx activities of heart homogenates from rats given the standard diet (○), Se(−) diet (△), and Se(−) diet + γ-GCE (□) were determined with variable GSH concentrations. Each point represents the average value of triplicate experiments, and the deviation of each point was within 5% of the average.

Effects of Se Deficiency and γ-GCE Administration on TBARS Levels in 14-day-old Rat Hearts

The effects of dietary Se deficiency, Na_2SeO_3 supplementation (1 μg/g Se(−) diet), and γ-GCE administration (350 μmol/g body weight) on TBARS levels of hearts taken from 14-day-old rats are shown in Table 4. TBARS levels (malondialdehyde generated per mg protein) were measured in this study as an index of lipid peroxidation. The TBARS levels of 14-day-old rats given the Se(−) diet were about 22% to 30% higher than those given the standard diet, Se(−) diet + Na_2SeO_3, or Se(−) diet + γ-GCE ($P < 0.05$). This result indicated that TBARS generation increased in response to Se depletion; it also suggested that a high TBARS level in heart tissue may lead to cardiac dysfunction.

Effects of Se Deficiency and γ-GCE Administration on $[GSH]_i$ and Se-Gpx Activity in Fetal Rat Heart Homogenates

The $[GSH]_i$ was measured in heart homogenates prepared from 14-day-old fetuses taken from variously treated dams. The level was about 1.5 times higher when the dam had

TABLE 4. Effects of γ-GCE and Na_2SeO_3 on TBARS of hearts taken from 14-day-old Se(−) rats

Diet	TBARS (pmol MDA/mg protein)
Standard	653 ± 60
Se(−)	842 ± 31‡
Se(−) + Na_2SeO_3*	586 ± 68
Se(−) + γ-GCE injection†	625 ± 91

Results are expressed as means ± SD of three experiments.

ANOVA: $P < 0.05$ standard diet vs Se(−) diet.

*Na_2SeO_3 supplementation (1 μg/g of diet).

†Subcutaneous injection with γ-GCE (350 μmol/g weight) twice a day.

‡$P < 0.05$ by LSD.

TABLE 5. Effects of Se(−) + γ-GCE on $[GSH]_i$ and Se-Gpx activity of fetal rat heart homogenates

Maternal diet	$[GSH]_i$ (nmol/mg protein)	Maximum Se-Gpx activity (unit/mg protein/min)
Standard	5.2 ± 1.1	15 ± 0.8
Se(−)	4.8 ± 1.5	8 ± 1.3‡
Se(−) + γ-GCE injection*	7.8 ± 1.1†	9 ± 1.5§

$[GSH]_i$ indicates the intracellular concentration of total (oxidized and reduced) glutathione.

ANOVA: $P < 0.1$ standard diet vs Se(−) diet + γ-GCE in $[GSH]_i$; $P < 0.001$ standard diet vs Se(−) diet in maximal Se-Gpx activity; and $P < 0.001$ standard diet vs Se(−) diet + γ-GCE in Se-Gpx activity. Results are expressed as means ± SD of four experiments.

*Subcutaneous injection with γ-GCE (350 μmol/g body weight of a rat) twice a day.

†$P < 0.05$ *by LSD*.

‡$P < 0.0005$ by LSD.

§$P < 0.005$ by LSD.

been given the Se(−) diet + γ-GCE than when it had been fed the standard or Se(−) diet ($P < 0.05$) (Table 5). There was no significant difference in $[GSH]_i$ in heart homogenates of fetuses from dams fed the standard diet and those fed the Se(−) diet. The maximum Se-Gpx activities in homogenates from the Se(−) diet and Se(−) diet + γ-GCE groups were significantly lower than in homogenates from the standard diet group. These observations indicated that the administration of γ-GCE to pregnant dams fed a Se(−) diet increased $[GSH]_i$ in fetal and neonatal heart tissue, but did not maximize Se-Gpx activity in the tissue. These observations may also indicate that the rest of the GSH maintenance system (reductase/synthetases, etc.) is unaffected by Se.

Resistance to H_2O_2 Toxicity of Cultured Rat Cardiac Myocytes

We next examined the protective effect of γ-GCE, Na_2SeO_3, and GSH on H_2O_2-induced contractile impairment of cultured fetal rat cardiac myocytes. Cardiac myocytes were isolated from fetuses of dams given the various treatments and then incubated in the presence of γ-GCE. The cells showed a higher $[GSH]_i$ when the dams had been given the Se(−) diet + γ-GCE than when they were given the standard or Se(−) diets (Table 6). The $[GSH]_i$ was first calculated as mmol/mg of protein and then as mmol/kg of cell water (assuming that 1 mg protein is equivalent to 5.1 mg of cell water) [29], and the $[GSH]_i$ in the standard diet, Se(−) diet, and Se(−) diet + γ-GCE group was estimated to be 1.7, 1.9, and 3.4 mM, respectively. These values were considerably lower than the apparent Se-Gpx K_m for GSH (11–12 mM) in the heart homogenates (see Table 3). The cardiac myocytes isolated from fetuses of rats fed the Se(−) and standard diets showed normal spontaneous beating in the presence or absence of either γ-GCE or Na_2SeO_3. On addition of 50 μM H_2O_2, the beating rate increased and then progressively decreased. After the spontaneous beating stopped, electrical stimulation elicited beating for a short period of time until finally, the cells did not respond to the stimulation. Figure 3 showed the mean time to cessation of both spontaneous and stimulation-elicited beating after addition of 50 μM H_2O_2. Myocytes isolated from fetuses taken from Se-deprived dams, relative to those isolated from fetuses taken from dams fed a standard diet, were more susceptible to H_2O_2 toxicity. The difference in susceptibility disappeared when the Se-deficient myocytes were incubated for 20 hr in medium containing 1 μM Na_2SeO_3 (Fig. 3). Incubation in medium containing 200 μM Na_2SeO_3 for 1 hr, however, increased the difference. Myocytes that were isolated from fetuses taken from Se-deficient but γ-GCE-treated rats and then incubated in the presence of 500 μM γ-GCE were considerably more resistant to H_2O_2 toxicity than myocytes from any other treatment group. Incubation of Se-deficient myocytes for 20 hr in the presence of GSH did not enhance H_2O_2 resistance (Fig. 3), nor did a prospective catalytic substance after de-esterification of γ-GCE, ethyl alcohol (0.1–2 mM).

These observations suggested that an increase in $[GSH]_i$ can protect the cultured rat cardiac myocytes against H_2O_2 toxicity.

DISCUSSION

Cardiac Dysfunction Induced by Se(−) Diet and Possible Mechanisms

In the present work, we found that a Se(−) diet fed to pregnant rats caused electrical and mechanical abnormalities in the heart tissue of their newborn offspring (Fig. 1 and

TABLE 6. Effects of Se(−) diet and γ-GCE on $[GSH]_i$ of cultured fetal rat cardiac myocytes

Maternal diet	γ-GCE (mM) in culture medium	$[GSH]_i$ (nmol/mg protein)	$[GSH]_i$ (mM)
Standard	—	8.9 ± 1.8	1.7†
Se(−)	—	9.8 ± 1.0	1.9
Se(−) + γ-GCE injection*	500	17.5 ± 1.8‡	3.4

$[GSH]_i$ indicates the intracellular concentration of total (oxidized and reduced) glutathione. The $[GSH]_i$ differs among the three groups (one-way ANOVA, $P < 0.05$). Results are expressed as means ± SD of three experiments.

*Subcutaneous injection with γ-GCE (350 μmol/g body weight) twice a day.

†$[GSH]_i$ was first calculated as mmol/mg of protein and then as mmol/kg of cell water by assuming that 1 mg protein is equivalent to 5.1 mg of cell water.

‡Significantly different from standard diet (LSD post-ANOVA test, $P < 0.001$).

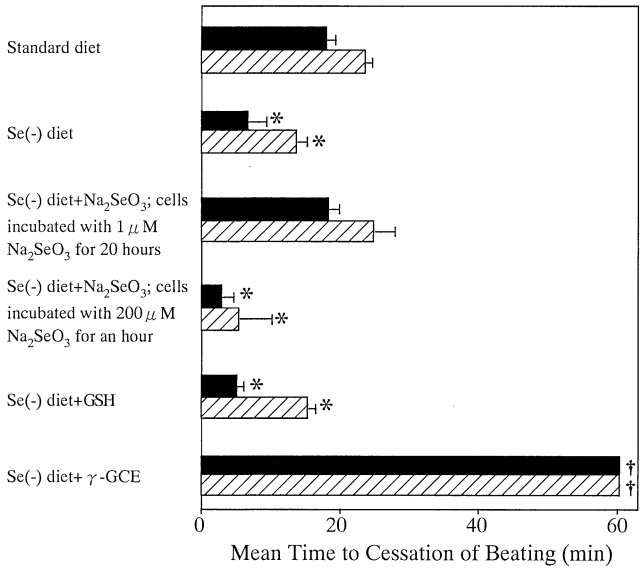

FIG. 3. Bar graphs showing the effects of γ-GCE, GSH, and Na_2SeO_3 on the H_2O_2 sensitivity of cultured fetal rat cardiac myocytes. Cells were prepared from fetuses taken on gestation day 14 from dams that had received four different treatments. Cells were preloaded with γ-GCE, GSH, or Na_2SeO_3 for 20 hr and with 200 μM Na_2SeO_3 for 1 hr in serum-, methionine- and cysteine-free Eagle's minimum essential medium. They were then exposed to 50 μM H_2O_2 in the absence of γ-GCE, GSH, and Na_2SeO_3. Solid bars and hatched bars represent mean time to onset of the cessation of spontaneous beating and the cessation of electrical stimulation-elicited beating, respectively. Results are expressed as means ± SD of five experiments. ANOVA: $P < 0.0001$ standard diet vs Se(−) diet, Se(−) diet + Na_2SeO_3 (incubated with 200 μM Na_2SeO_3 for 1 hr), or Se(−) diet + GSH (incubated with 500 μM GSH for 20 hr), *$P < 0.0001$ by LSD. †During an observation period of 60 min, no cessation of spontaneous or stimulation-elicited beating occurred in the Se(−) diet + γ-GCE (incubated with 500 μM γ-GCE for 20 hr).

Table 2). No significant ultrastructural lesions, however, were observed in the LV of hearts showing ECG abnormalities. Patients receiving long-term total parenteral nutrition (TPN) therapy develop Se deficiency [30, 31], and their ECGs show abnormalities such as arrhythmia [31, 32] and multiple ventricular extrasystole [32, 33]. This is in agreement with our present results in rats. Acute Keshan disease, a cardiomyopathy endemic in China, is characterized by heart failure with arrhythmia and cardiogenic shock [30], while chronic Keshan disease is characterized by severe cardiomyopathy, usually with congestive heart failure and a varying degree of pathological changes [32]. Thus, diets that provide only negligible amounts of Se may induce cardiac dysfunction. Patients with TPN-associated Se deficiency showed no characteristic signs and symptoms except for low serum Se-Gpx activity [31]. This, too, is supported by our observation that rats fed the Se(−) diet were normal in appearance and exhibited no pathology. Se is used as an adjuvant to antiarrhythmic therapy [34], and Se-containing compounds exhibit an antiarrhythmic effect in experimental animals [35]. Thus, Se may be important to normal cardiac function.

In an earlier study, Se deficiency alone did not lead to abnormal cardiac function in the rat, but in combination with vitamin E deficiency, it did [36]. This study conflicts with ours, but the conflict may be due to differences in the age of the animals and the condition of their cellular antioxidants and antioxidant enzymes. For example, the intravenous oxygen partial pressure for fetal, neonatal, and adult rats was 11 mmHg [37], 38 to 48 mmHg [38], and 84 to 87 mmHg [39], respectively. Se-Gpx activity is low in fetal hearts and rises after birth, and the TBARS level is higher in fetal than in maternal tissues because of their less-developed antioxidant defense mechanisms [40]. In addition, there is evidence that serum Se levels are lower in infants than in adults and that Se-Gpx activity parallels tissue Se levels [41]. Immature animals, including neonatal rats and humans, therefore, may be more sensitive to oxidative stress than adults. This may be why Se deficiency induced cardiac dysfunction more readily in neonatal than in adult rats.

An explanation of the mechanism by which Se deficiency induces cardiac dysfunction should consider the following: (1) Se is a component of Se-Gpx, which catalyzes the reduction of hydrogen and lipid peroxides (the products of reactive oxygen species) via oxidation of GSH [4, 5]. Se deficiency would lead to low Se-Gpx activity, and that in turn would lead to an accumulation of lipid peroxides (Table 4). In hearts, Se deficiency enhances the accumulation of lipid peroxidation following ischemia–reperfusion injury [42], oxygen exposure [43], and therapeutic administration of anthracycline anticancer drugs [44, 45]; and (2) Enhanced lipid peroxidation, in turn, may involve cell membrane damage, which could lead to cardiac dysfunction and an increase in intracellular free calcium concentration (calcium overload) by impairment of the calcium transport system. Calcium overload induces irreversible cardiac injury [46, 47].

Protective Effects of $[GSH]_i$ against Se(−)-induced Heart Dysfunction

In the present work, we tested whether Se(−)-induced cardiac dysfunction could be lessened by an increase in $[GSH]_i$. We used a membrane-permeating GSH precursor, γ-GCE [17–20], because GSH itself does not penetrate the cell membrane [17, 21]. We found that subcutaneous administration of γ-GCE, but not GSH, to Se(−) rats increased cardiac $[GSH]_i$ (Table 3), and that the rats with a high $[GSH]_i$ were much more resistant to Se(−)-induced cardiac dysfunction (Table 2 and Fig. 1). The TBARS level was also low in heart tissue containing high $[GSH]_i$ (Table 4). In addition, preloading of cultured rat cardiac myocytes with γ-GCE increased both $[GSH]_i$ and H_2O_2 resistance. These observations indicate that Se(−)-induced cardiac dysfunction was reduced by an increase in $[GSH]_i$, and that inhibition of the Se(−)-induced increase in TBARS by the increase in $[GSH]_i$ may have contributed to the improved function.

The next question we posed was how GSH improved the cardiac function of Se-deficient rats. Kinetic analysis of Se-Gpx (Fig. 2 and Table 3) showed that the Se(−) diet decreased the V_{max} of Se-Gpx activity in the Se(−) hearts, but not the K_m for GSH, and that administration of γ-GCE did not affect the V_{max} of Se-Gpx activity or the K_m for GSH. The kinetic study showed the K_m value for GSH to be 11–12 mM, which was considerably higher than the concentration of GSH in cardiac myocytes. The $[GSH]_i$ in cardiac myocyte-isolated Se(−) rats was calculated to be 1.9 mM, while the value for the Se(−) diet and Se(−) diet + γ-GCE groups was calculated to be 3.4 mM. Our kinetic study indicates that if $[GSH]_i$ were to increase from 1.9 mM to 3.4 mM, the velocity of H_2O_2 decomposition by Se-Gpx would increase 1.6-fold (Fig. 2). The rapid elimination of intracellular H_2O_2 via Se-Gpx and/or other radical derivative components, such as OH·, may cause a decrease in TBARS level and an improvement of Se(−)-induced dysfunction. This speculation, however, did not exclude the possibility that GSH directly protected against the radical-induced toxicity in cardiac myocytes.

Changes in $[GSH]_i$ may affect the heart's function and antioxidant capacity. GSH-deficient hearts subjected to 30 min of normoxic perfusion exhibit an accelerated temporal decay of contractile function [48]. The recovery of post-ischemic contractile function is depressed in GSH-depleted hearts [49]. Furthermore, Na^+/K^+ ATPase activity may be closely coupled to myocardial $[GSH]_i$, and the inhibition of Na^+/K^+ ATPase activity during ischemia–reperfusion injury may contribute to calcium accumulation and its attendant hazards, such as reperfusion-induced arrhythmias [50]. These observations suggest that cellular GSH plays a key role in maintaining normal cardiac function. In addition, GSH is a cofactor for H_2O_2-mediated stimulation of Ca^{2+}-induced Ca^{2+} release from sarcoplasmic reticulum in rat cardiac myocytes [51], suggesting that GSH is a signal transducer for cardiac muscle excitation–contraction coupling. Thus, the beneficial effects of increased $[GSH]_i$ as described above may explain why γ-GCE plays an active protective role against Se(−) induced cardiac dysfunction and H_2O_2 toxicity.

In 1979, Chinese scientists carried out an extensive Na_2SeO_3 supplementation program to prevent Keshan disease [14]. Indeed, Se is essential at trace concentrations (40 μg/day) and toxic at high concentrations (400–600 μg/day) [52]. Selenosis occurs in parts of Enshi region where food has a high Se content. Symptoms appeared at Se intakes ≧910 μg/day, corresponding to blood levels ≧1.05 mg/L [53]. Thus, Se itself is not always safe to administer, and our present data showed that addition of Na_2SeO_3 ≧200 mM decreased the H_2O_2 resistance of cultured cardiac myocytes (Fig. 3). Hence, it might be more beneficial to administer γ-GCE to prevent cardiac dysfunction induced by dietary Se depletion.

In this study, we used a torula yeast-based semisynthetic Se(−) diet that contained vit. E (10 mg/100 g of Se(−) diet). Because vit. E deficiency and coxsackie virus B are needed in addition to Se deficiency for the development of the severe cardiomyopathy and ultrastructural lesions characteristic of chronic Keshan disease [32]. We plan to study whether an increase in $[GSH]_i$ by administration of γ-GCE can reverse the cardiac dysfunction induced by these three factors working in concert.

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